

Inhibition of Phosphodiesterase 4 Attenuates Airway Hyperresponsiveness and Airway Inflammation in a Model of Secondary Allergen Challenge

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We compared for the first time the therapeutic potential of a specific phosphodiesterase 4 (PDE4) inhibitor, rolipram, with anti-VLA-4 and anti-IL-5 in a model of secondary allergen exposure of previously sensitized and challenged mice. To address these issues, mice were sensitized and challenged with ovalbumin (OVA) (primary challenge). Six weeks later, sensitized/challenged mice were reexposed to OVA (secondary challenge) and airway response (resistance [R_L] and dynamic compliance [C_{dyn}]) to inhaled methacholine was monitored. After secondary OVA challenge, R_L significantly increased as did the number of lung inflammatory cells and IL-4 and IL-5 production in bronchoalveolar lavage fluid (BALF). Administration of rolipram, in a dose-dependent manner, significantly prevented both changes in R_L and C_{dyn} , as well as eosinophil, lymphocyte, and neutrophil accumulation in the BALF; IL-4 and IL-5 levels in BALF were also significantly reduced. In contrast, treatment with anti-VLA-4 and anti-IL-5 only prevented changes in R_L and eosinophil numbers and IL-5 production in BALF. Further, goblet cell hyperplasia was suppressed only by treatment with rolipram. None of the treatments affected OVA-specific antibody levels. These studies confirm that IL-5 dependent eosinophilic inflammation plays an essential role in the development of certain aspects of airway function after rechallenge of sensitized mice and that lymphocytes and neutrophils are also important in the development of altered airway function. The use of agents that inhibit PDE4 may have an important role in the treatment of asthma in previously sensitized mice.

Bronchial asthma is a syndrome associated with allergen-induced airway hyperresponsiveness (AHR) and chronic airway inflammation. Airway mucosal inflammation is characterized by an influx of activated eosinophils and T lymphocytes (1). The pathophysiology of AHR is complex, and many independent factors contribute to its development. However, eosinophils are thought to be a major effector cell in the development of increased airway reactivity by releasing eosinophil granule proteins such as major basic protein (MBP) and eosinophilic cationic protein (ECP), and other mediators, including leukotrienes, that damage the airway epithelium and induce airway smooth muscle contraction and vascular leakage. The selective accumulation of these inflammatory cells at allergic inflammatory sites depends on the interactions between adhesion molecules on the infiltrating cells and endothelial cells, and a number of cytokines and chemokines.

In particular, the very late activating antigen-4 (VLA-4) and IL-5 appear to be important in aspects of the pathogenesis

of the disease. Because VLA-4 is expressed on the surface of eosinophils and T lymphocytes (2), this receptor may play an important role in the selective entry of eosinophils and T lymphocytes, but not neutrophils, into inflamed tissues in asthma. Recently, we reported that VLA-4 mAb inhibited not only the migration of eosinophils into the airways but also allergen-induced AHR (3). IL-5, on the other hand, is believed to be a key cytokine in orchestrating eosinophilic inflammation. The infiltration of eosinophils into the airways has been linked to the production of IL-5, which is important for eosinophil proliferation, activation, and migration. The number of eosinophils in bronchoalveolar lavage fluid (BALF) and lung biopsies has correlated with the severity of asthma in patients (4). Increases in IL-5 levels in BALF and IL-5 mRNA expression in BALF cells and lung tissues are also observed in asthmatic patients after allergen inhalation challenge. Genetic deficiency of IL-5 (5) or treatment with anti-IL-5 (6) prevents the infiltration of eosinophils and the development of AHR in murine models.

Cyclic nucleotides, particularly cyclic AMP (cAMP), have important regulatory roles in all cell types involved in the pathophysiology of asthma, because cAMP broadly suppresses the activity of immune and inflammatory cells. Intracellular cAMP levels reflect the balance between the generation of cAMP from ATP by adenylate cyclase and its degradation by cyclic nucleotide phosphodiesterase (PDE), which is a multigene enzyme family with distinct characteristics. Elevation of intracellular cyclic nucleotide levels via the inhibition of PDE might induce bronchodilation and inhibit pulmonary inflammation. At present, *at least nine* PDE isoenzyme family members have been identified (7). In particular, PDE4, cAMP-specific PDE, is expressed in a variety of inflammatory cells, including eosinophils, neutrophils, mast cells, and T cells. The effects of PDE4 inhibitors in models of pulmonary inflammation have been evaluated, but exclusively in primary challenge models. The most impressive property of PDE4 inhibitors is their ability to abolish antigen-induced eosinophil infiltration in guinea pigs (8, 9), rabbits (10), monkeys (11), and rats (12). Several reports demonstrated the effects of PDE4 inhibitors on AHR in guinea pigs (9) and monkeys (11). PDE4 inhibitors suppressed PAF- and C5a-stimulated LTC₄ production from peripheral blood eosinophils of atopic patients (13). IL-4 and IL-5 as well as IL-13 production from antigen-stimulated T-cell clones derived from atopic subjects was suppressed by treatment with a PDE4 inhibitor (14). Moreover, PDE4 is the predominant cAMP-metabolizing enzyme in neutrophils and eosinophils and PDE4 inhibitors reduce superoxide anion production from peripheral blood neutrophils (15, 16). The pulmonary vasculature and endothelium are another potential site of action because endothelial cells express PDE4, and increased levels of cAMP in these cells have been shown to reduce the expression of the VCAM-1 and ELAM-1 (17). Here, we investigated the effects of a PDE4 inhibitor on allergen-in-

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duced AHR and inflammatory cell infiltration in the airways in a secondary allergen challenge model. We demonstrate the significant inhibitory effects of a PDE4 inhibitor after secondary challenge, effects on airway inflammation and airway function, which extend beyond those observed after either anti-VLA-4 or anti-IL-5 administration.

METHODS

Animals

Female BALB/c mice from 8 to 12 wk of age were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were maintained on diets free of ovalbumin (OVA). All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

PDE4 Inhibitor and Monoclonal Antibody

Rolipram was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in ethanol and diluted with saline. The final concentration of ethanol was less than 1%. Mice received an intraperitoneal injection of rolipram 0.03, 0.1, and 0.3 mg/kg 2 h before and 6 and 24 h after OVA provocation (Figure 1). As a control, mice were administered saline intraperitoneally.

The rat antimouse VLA-4 mAb, PS/2 was purified from the hybridoma (American Type Culture Collection, Manassas, VA) using a protein G-sepharose affinity column (Pharmacia, Uppsala, Sweden) under endotoxin-free conditions. Antimouse IL-5 mAb was purified from supernatants of hybridoma cells (TRFK-5) (6) by protein-G separation. Mice received a single intravenous injection of rat anti-VLA-4, rat anti-IL-5 (TRFK-5) or rat IgG (Sigma) 2 mg/kg as control 2 h before OVA provocation (Figure 1).

Sensitization, Airway Challenge, and Allergen Provocation

Mice (six to eight mice/group/experiment) receiving the following treatment were studied. (1) airway challenge after nebulization of OVA alone in nonsensitized animals (N group); (2) intraperitoneal sensitization with OVA and OVA airway challenge (IPN group); (3) intraperitoneal sensitization, airway challenge with OVA, and OVA provocation (secondary challenge) via the airway (IPN/Provo group). Mice were sensitized by intraperitoneal injection of 20 μ g of OVA (Grade V; Sigma) emulsified in 2.25 mg alum (AlumImject; Pierce, Rockford, IL) in a total volume of 100 μ l on Days 0 and 14. Mice were challenged via the airways by OVA (1% in saline) for 20 min on Days 28, 29, and 30 by ultrasonic nebulization (particle size, 1–5 μ m; DeVillbiss, Somerset, PA). Six weeks after the last of the three primary OVA challenges, mice were exposed to 1% OVA for 20 min by nebulization (secondary challenge) (Figure 1). Airway function was assessed on Days 2, 7, 14, and 6 wk after the last of the three primary allergen challenges for N and IPN groups and at 48 h after 1% OVA provocation for IPN/Provo group, and the mice were killed to obtain tissues and cells for further assay.

Determination of Airway Resistance and Dynamic Compliance

Airway resistance (R_L) and dynamic compliance (C_{dyn}) were determined as a change in airway function after aerosolized methacholine (MCh) challenge. Anesthetized, tracheostomized mice were mechanically ventilated, and lung function was assessed as described (18). A four-way connector was attached to the tracheostomy tube (stainless steel cannula, 18G), with two ports connected to the inspiratory and expiratory sides of two ventilators. Ventilation was achieved at a rate of 160 breaths/min, tidal volume of 150 μ l with a positive end-expiratory pressure of 2 to 3 cm H_2O by the ventilator (Model SN-480-7-3; Shinano Manufacturing Co., Tokyo, Japan). Aerosolized MCh was administered for 10 breaths at a rate of 60 breaths/min, tidal volume (V_T) of 500 μ l by the ventilator (Model 683; Harvard Apparatus, South Natick, MA) in increasing concentrations (1.56, 3.125, 6.25, and 12.5 mg/ml). After each MCh challenge, the data were continuously collected for 1 to 5 min and maximum values of R_L and minimum values of C_{dyn} were taken to express changes in these functional parameters.

Bronchoalveolar Lavage and Measurement of BALF Cytokines

After assessment of R_L and C_{dyn} , lungs were lavaged via the tracheal tube with Hanks' balanced salt solution (HBSS, 1×1 ml, 37° C). The volume of collected BALF was measured in each sample and the number of BAL cells was counted by cell counter (Coulter Counter; Coulter Co., Hialeah, FL). Cytospin slides were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated in a blinded fashion by counting at least 300 cells under light microscopy.

Cytokine levels in the BALF supernatants were measured by ELISA as described (6). Cytokine levels were determined by comparison with the known standards. The limits of detection were 4 pg/ml.

Measurement of Serum Anti-OVA Antibody and Total Ig Levels

Anti-OVA IgE and IgG₁ antibody levels were measured by ELISA as previously described (18), 48 h after the last airway challenge. The antibody titers of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units per milliliter (EU/ml). Total IgE level was determined using the same method compared with a known mouse IgE standard (PharMingen, San Diego, CA). The limit of detection was 100 pg/ml for IgE.

Histologic and Immunohistochemistry Studies

After obtaining the BALF, lungs were inflated through the tracheal tube with 2 ml air and fixed in 10% formalin. Blocks of lung tissue were cut around the main bronchus and embedded in paraffin blocks. Tissue sections 5 μ m thick were affixed to microscope slides and deparaffinized. The slides were stained with hematoxylin-eosin and peri-

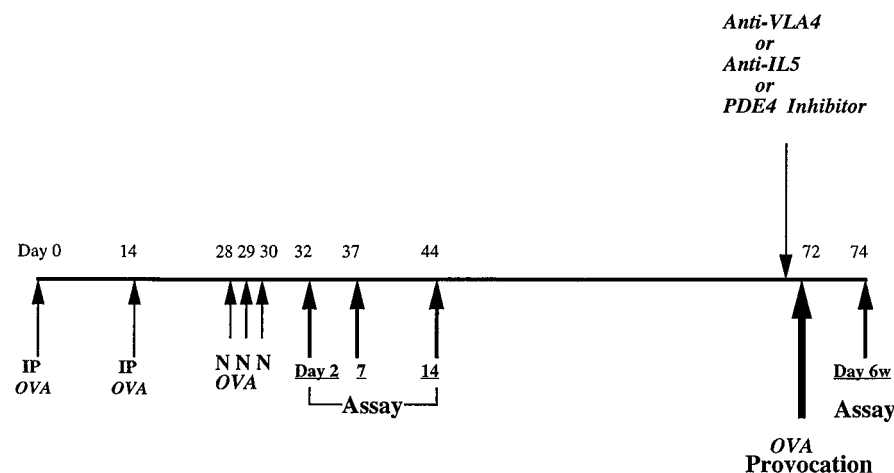


Figure 1. Schematic representation of the sensitization, challenge and antibody treatment protocol. Mice were sensitized intraperitoneally on Days 0 and 14 with OVA, and received three (primary) challenges with OVA on Days 28, 29, and 30 by ultrasonic nebulization. Six weeks after the last of the three primary OVA challenges, mice were exposed to 1% OVA for 20 min by nebulization (secondary challenge). Airway function was assessed on Days 2, 7, and 14, and 6 wk after the last of the three primary allergen challenges and at 48 h after 1% OVA provocation (secondary challenge). Mice received a single intravenous injection of anti-VLA-4 or anti-IL-5 2 h before OVA provocation, or an intraperitoneal injection of rolipram 0.03, 0.1 and 0.3 mg/kg 2 h before and 6 and 24 h after OVA provocation.

odic acid Schiff (PAS) for identification of mucus containing cells (19), and examined under light microscopy.

Cells containing MBP in lung sections were identified by immunohistochemical staining as described using a rabbit antimouse MBP (provided by Dr. J. J. Lee, Scottsdale, AZ) (6). The slides were examined in a blinded fashion with a Zeiss microscope equipped with a fluorescein filter system. Numbers of eosinophils in the peribronchial and perivascular tissue were analyzed using the IPLab2 software (Signal Analytics, Vienna, VA) for the Macintosh counting four different sections per animal (6).

The numbers of goblet cells in the airway epithelium were counted in at least 20 sections by measuring the length of epithelium defined along the basement membrane and the luminal area using the NIH Image Analysis system. Mucus containing cells were expressed as the number of goblet cells per 100 μ m epithelium.

Isolation and Analysis of Lung Lymphocytes

Lung cells were isolated as previously described (20). Lungs were perfused with warmed (37° C) calcium- and magnesium-free HBSS containing 10% FCS, 0.6 mM EDTA, 100 U/ml penicillin, and 100 μ g/ml streptomycin via the right ventricle at a rate of 4 ml/min for 4 min. Lungs were removed and minced. The minced lung tissues were then subjected to enzymatic digestion in an orbital shaker in a 37° C incubator for 1 h, using 4 ml HBSS containing 175 IU/ml collagenase (type IA; Sigma), 0.01% DNase (type I; Sigma), 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The digested lungs were sheared with a sterile 20-gauge needle and filtered through 45- and 15- μ m filters. Filters were washed with HBSS/2% FCS. After collecting the cells by centrifugation, mononuclear cells were purified by passing the tissue through a stainless steel mesh, followed by density-gradient centrifugation (Organon Teknika, Durham, NC). The cells were re-

suspended in HBSS and counted with a hemocytometer and plated in 96-well round-bottom plates at 400,000 cells/well. After preincubation with mouse serum, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated antimouse CD3 (145-2C11), B220 (RA3-6B2) or phycoerythrin (PE)-conjugated antimouse CD4 (RM4-5), CD8 (53-6.7) (Pharmingen). After washing, cells were examined (10,000 gated events were analyzed) using an EPICS XL analyzer (Coulter Electronics, Hialeah, FL). Results are expressed as the percentage of cells expressing a given surface marker.

Statistical Analysis

Values for all measurements are expressed as the mean and standard error of the mean (SEM). Student's two-tailed unpaired *t* test was used to determine the levels of difference between two experimental groups. ANOVA was used to compare percent changes in *R*_L and *C*_{dyn} between different groups with the same treatment. The *p* values for significance were set at *p* < 0.05.

RESULTS

Secondary Challenge with OVA Induces Marked Increases in *R*_L and Inflammatory Cell Infiltrates

OVA sensitized and nonsensitized BALB/c mice were challenged with an aerosol of OVA on three consecutive days. We assessed airway responsiveness to inhaled MCh on Days 2, 7, and 14, and 6 wk after the last allergen challenge. The response to MCh in (nonsensitized) mice challenged with OVA alone showed small changes in *R*_L and in *C*_{dyn}. After OVA sensitization and challenge, AHR was demonstrated; *R*_L values were sig-

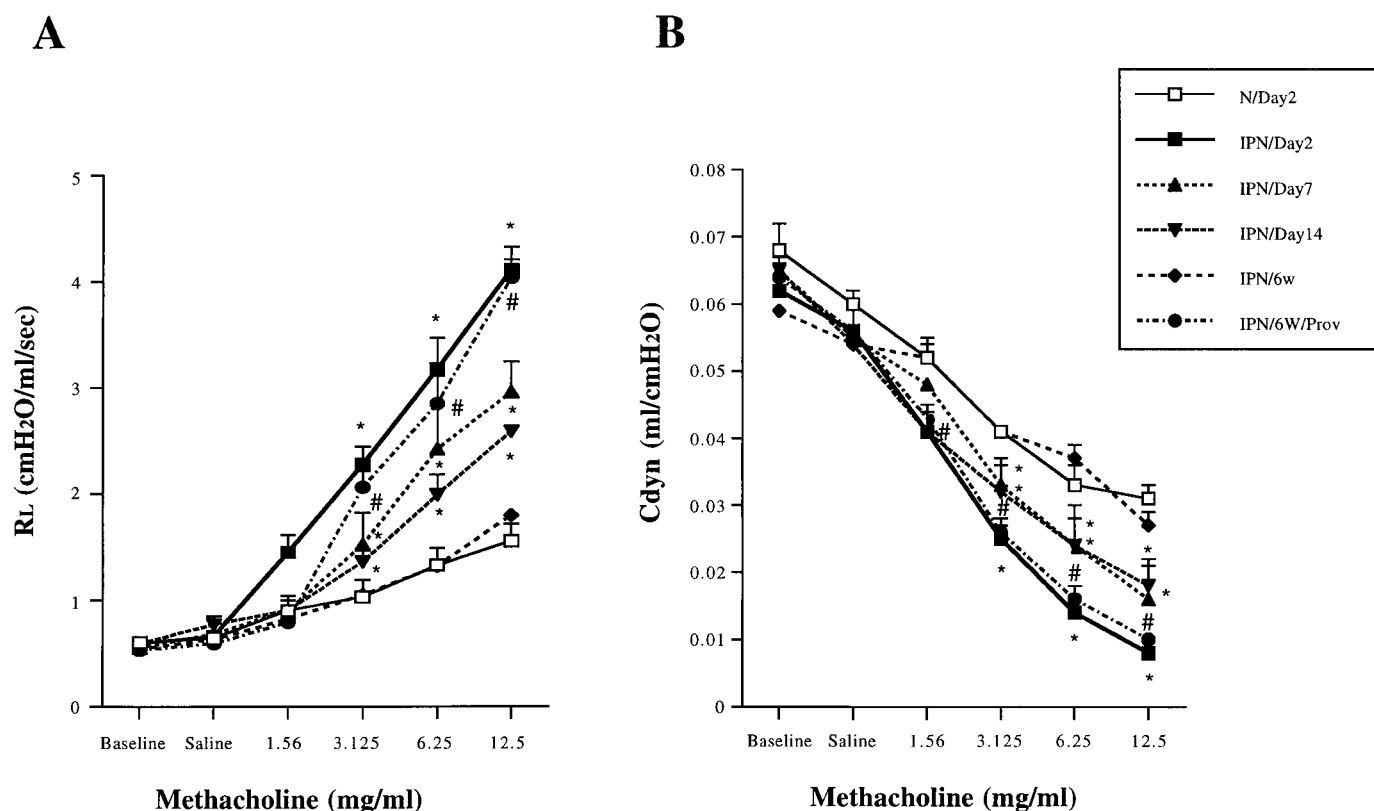


Figure 2. *R*_L (A) and *C*_{dyn} (B) in OVA challenge alone (N/Day 2), OVA sensitization and challenge (IPN/Days 2, 7 and 14 and 6 wk) and OVA provocation after OVA sensitization and challenge (IPN/6 wk/Prov). *R*_L and *C*_{dyn} values were obtained in response to increasing concentrations of MCh as described in METHODS. Data represent the mean \pm SEM (*n* = 8). #Significant differences (*p* < 0.05) between OVA provocation after OVA sensitization and challenge group (IPN/6 wk/Prov) and without provocation group (IPN/6 wk). *Significant differences (*p* < 0.05) between the IPN groups shown and corresponding nonsensitized but challenged groups (not shown).

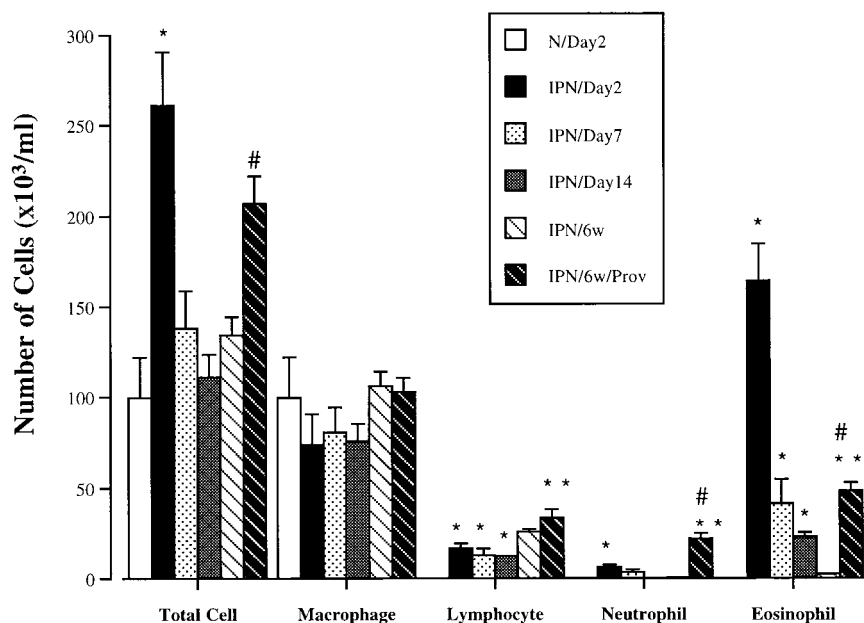


Figure 3. Cellular composition of BALF. Sensitization and subsequent challenge through the airways resulted in a significant increase in eosinophils and lymphocytes compared with challenge alone groups. Results of each group are expressed as the mean \pm SEM ($n = 8$). *Significant differences ($p < 0.05$) between OVA provocation after OVA sensitization and challenge group (IPN/6 wk/Prov) and without provocation group (IPN/6 wk). **Significant differences ($p < 0.05$) between OVA provocation after OVA sensitization and challenge group (IPN/6 wk/Prov) and 2 d after the last challenge group (IPN/Day 2). #Significant differences ($p < 0.05$) between the IPN groups shown and corresponding nonsensitized but challenged groups (not shown).

nificantly increased and Cdyn values significantly decreased in a dose-dependent manner (Figure 2). Significant changes in RL and Cdyn reached maximum levels 48 h (2 d) after the last challenge. Even 14 d after the last challenge, changes in RL and Cdyn persisted compared with controls. However, no significant changes in RL and Cdyn were seen 6 wk after the last challenge.

Numbers of eosinophils in the BALF paralleled the changes

in airway function (Figure 3). At 48 h after the last challenge, eosinophils comprised 60% of the total cell pool, by 14 d eosinophils represented 21% of the total cells, and few were detected at 6 wk. In contrast, the number of lymphocytes in BALF significantly increased during the 6 wk after challenge.

Single reexposure to OVA via the airways 6 wk after the last challenge (secondary challenge) resulted in AHR, with

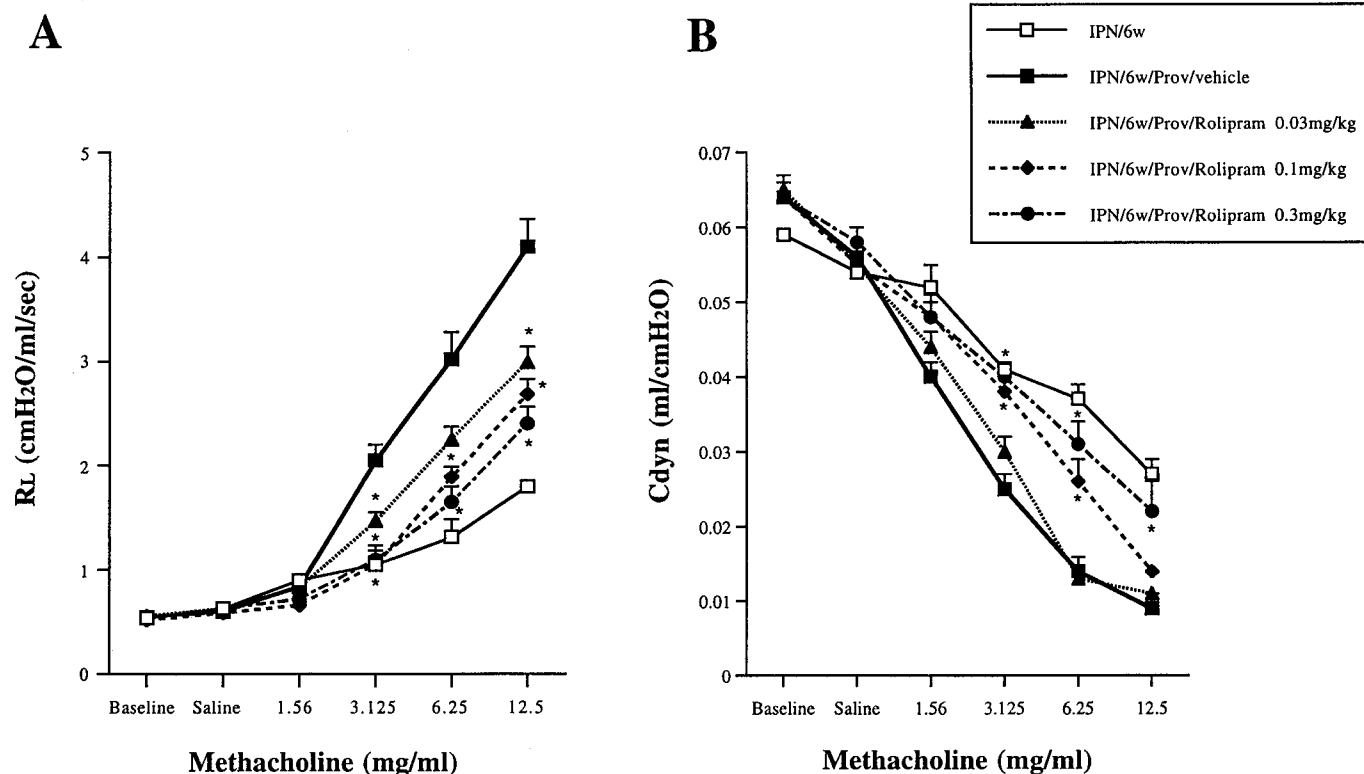


Figure 4. Treatment with rolipram prevents airway resistance (A) and Cdyn (B) after OVA provocation. Mice received an intraperitoneal injection of rolipram 0.03, 0.1, and 0.3 mg/kg (IPN/6 wk/Prov/Rolipram) or saline as vehicle (IPN/6 wk/vehicle) 2 h before and 6 h and 24 h after 1% OVA provocation after OVA sensitization and challenge. The results for each group are mean \pm SEM ($n = 8$). *Significant differences ($p < 0.05$) between OVA provocation and rolipram treated group and OVA provocation and saline-treated control group.

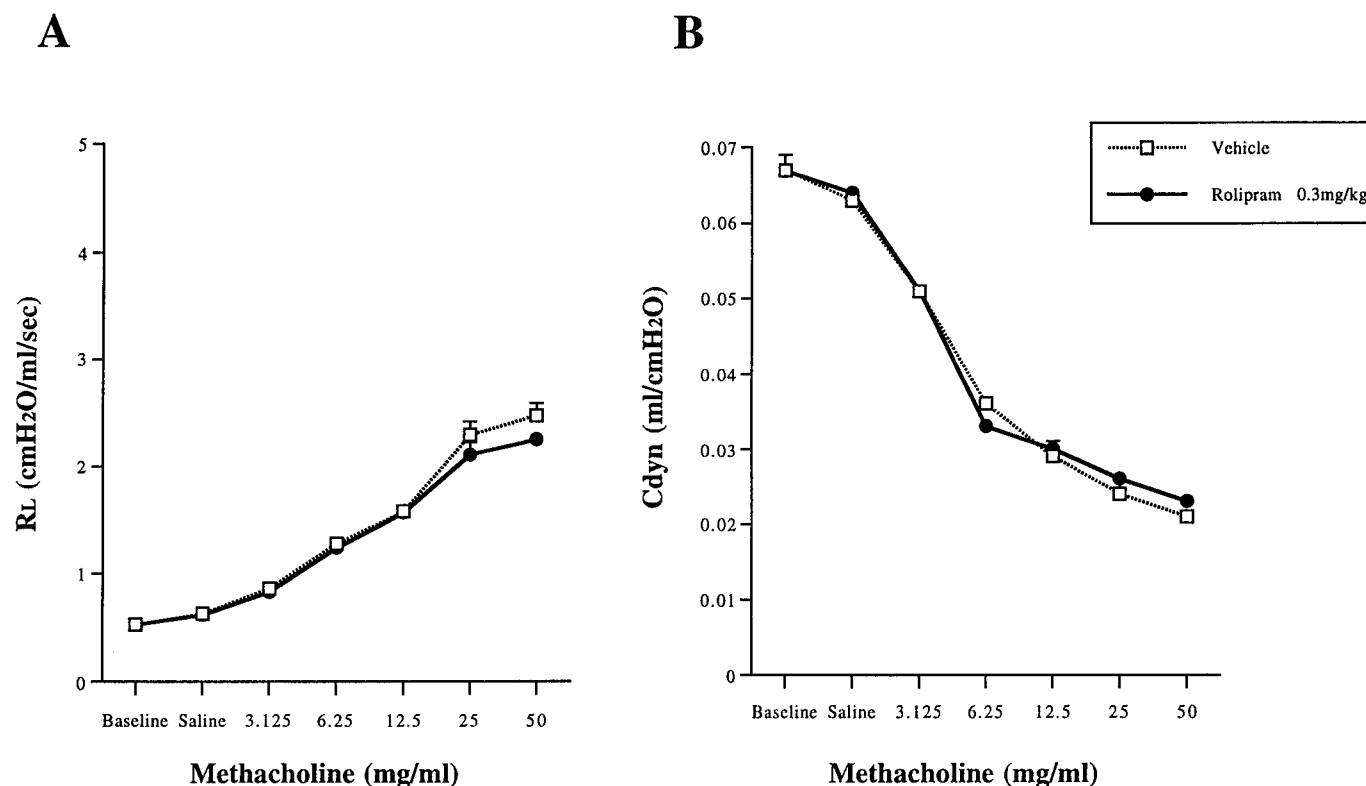


Figure 5. Influence of rolipram on MCh-induced bronchoconstriction in naive mice (A: airway resistance; B: dynamic compliance). Mice were treated with rolipram 0.3 mg/kg or saline as vehicle at three times and the development of changes in RL and Cdyn was evaluated. Results of each group are expressed as the mean \pm SEM (n = 6).

significant increases in RL and decreases in Cdyn, and induced a significant increase in numbers of inflammatory cells when compared with the absence of AHR 6 wk after the last challenge (without provocation) group (Figures 2 and 3) or provocation alone (data not shown).

Treatment with Rolipram Inhibits Airway Resistance and Cdyn after OVA Provocation

To determine the effect of the specific PDE4 inhibitor rolipram on the development of altered airway function (RL and Cdyn) after rechallenge with OVA, mice received an intra-

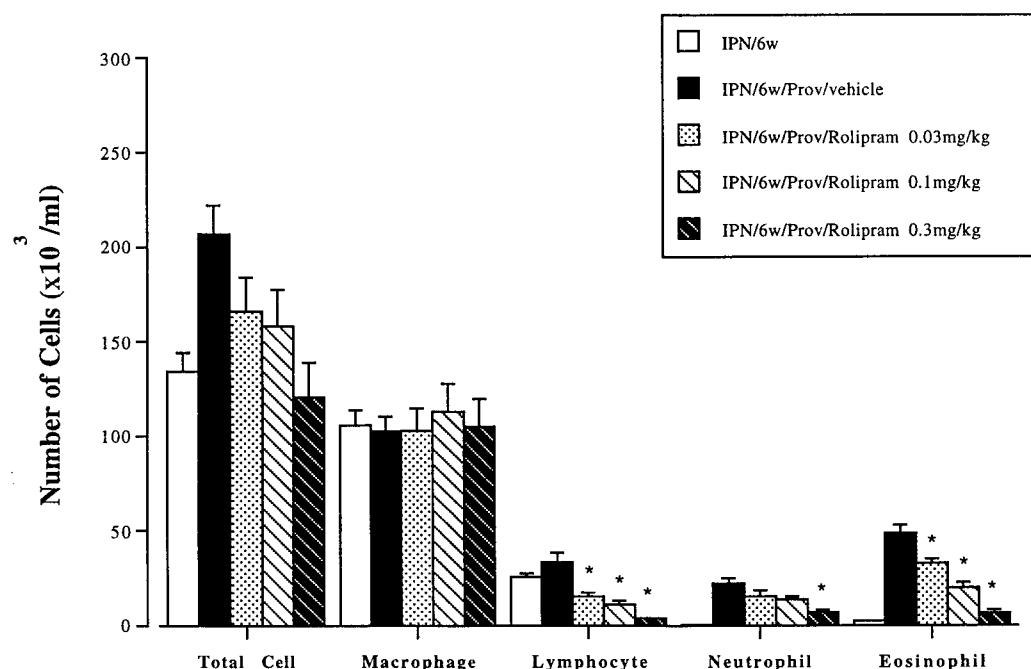


Figure 6. Effect of rolipram on inflammatory cell accumulation in BALF in mice receiving secondary OVA challenge. The number of inflammatory cells in BALF was determined 48 hrs after OVA provocation as described in METHODS. Data represent the mean \pm SEM (n = 8). *Significant differences (p < 0.05) between OVA provocation after OVA sensitization and challenge in rolipram treated mice and OVA provocation and saline-treated control mice.

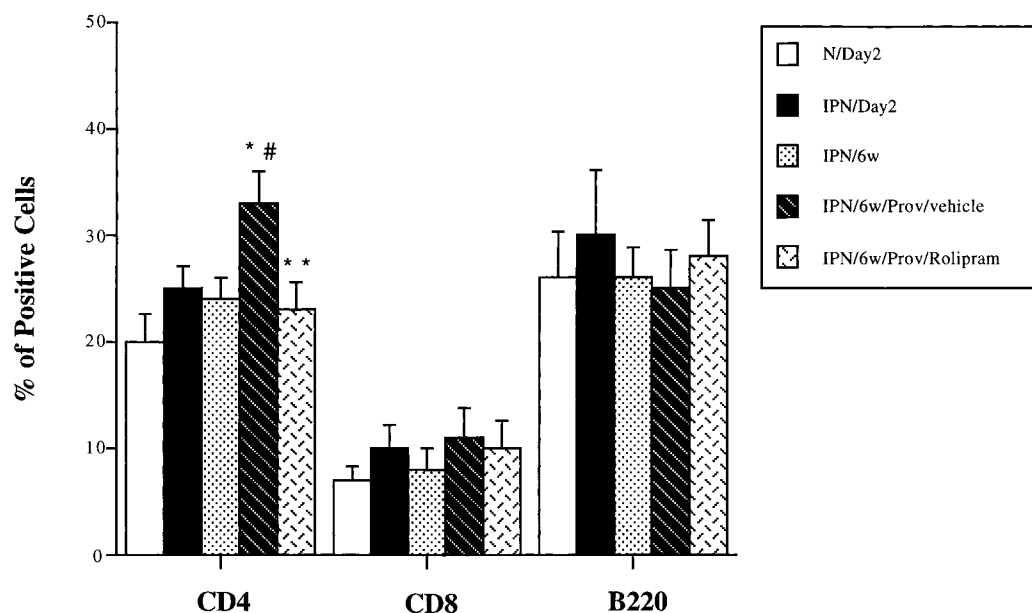


Figure 7. The percentage of lung mononuclear cells staining positive for CD4, CD8, and B220 was assessed after secondary challenge and rolipram treatment. The percentage of CD4+ cells in secondary-challenged mice was significantly increased and this increase was prevented by treatment with rolipram 0.3 mg/kg. Results from each group are expressed as the mean \pm SEM ($n = 6$). #Significant differences ($p < 0.05$) between OVA provocation control mice (IPN/6 wk/Prov/vehicle) and 2 d after the last challenge (IPN/Day 2). *Significant differences ($p < 0.05$) between OVA provocation control mice (IPN/6 wk/Prov/vehicle) and mice without provocation (IPN/6 wk). **Significant differences ($p < 0.05$) between saline-treated OVA provocation mice (IPN/6 wk/Prov/vehicle) and rolipram-treated OVA provocation mice (IPN/6 wk/Prov/Rolipram).

peritoneal injection of the drug (0.03, 0.1 or 0.3 mg/kg versus saline). Administration of rolipram significantly prevented the increases in R_L and reductions in C_{dyn} throughout the MCh dose-response curve in a dose-dependent manner (Figure 4).

Treatment with Rolipram Does Not Have Direct Effects On Airway Resistance and C_{dyn}

A direct bronchodilatory activity of PDE inhibitors has been demonstrated in some studies (21). To eliminate this possibil-

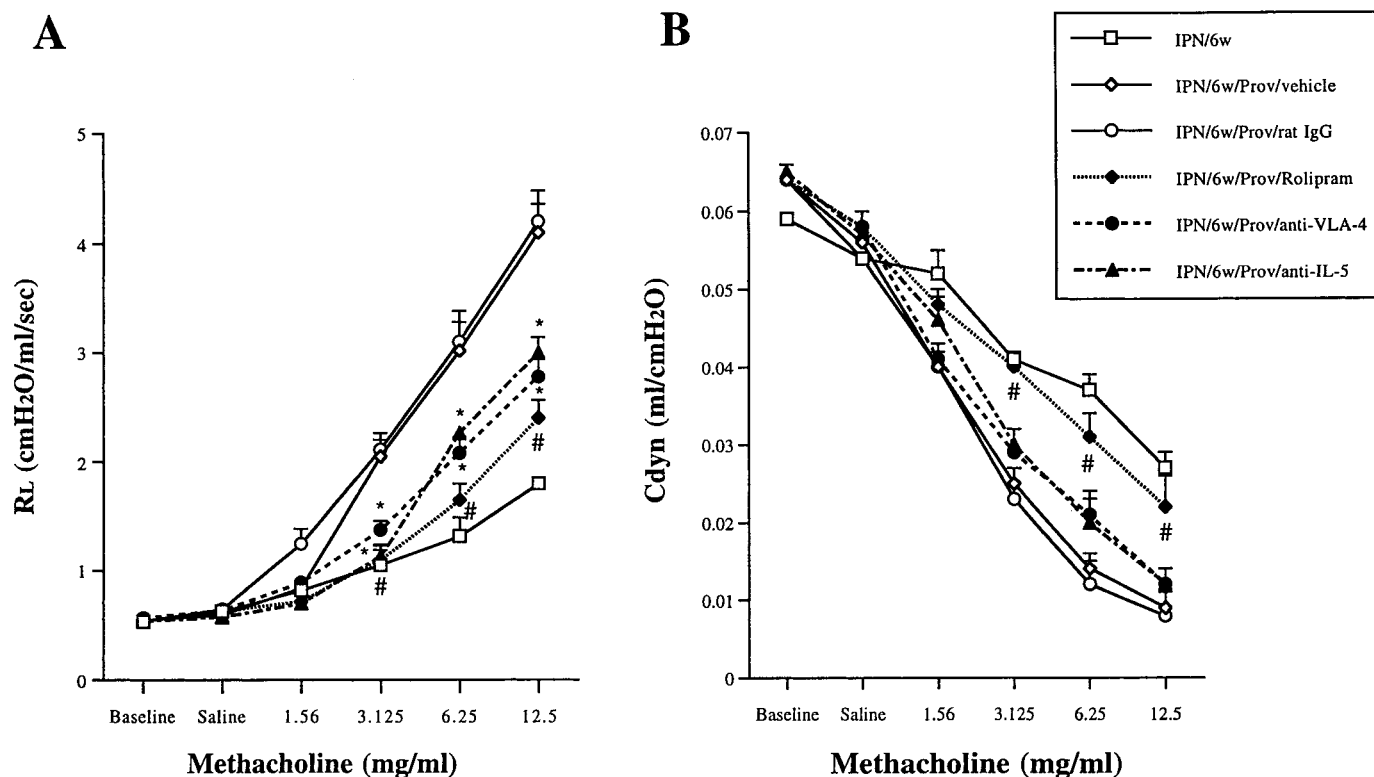


Figure 8. Anti-VLA-4 and anti-IL-5 significantly suppresses R_L but does not inhibit C_{dyn} in sensitized/challenged mice receiving secondary challenge with OVA. R_L (A) and C_{dyn} (B) was assessed 48 h after allergen provocation as described in METHODS. Mice received an intraperitoneal injection of rolipram 0.3 mg/kg (IPN/6 wk/Prov/Rolipram) or saline as vehicle (IPN/6 wk/Prov/vehicle) 2 h before and 6 h and 24 h after OVA provocation, or intravenous injection of anti-VLA-4 (IPN/6 wk/Prov/anti-VLA-4), anti-IL-5 (IPN/6 wk/Prov/anti-IL-5) or rat IgG as control (IPN/6 wk/Prov/rat IgG) 2 h before OVA provocation. The results for each group are mean \pm SEM ($n = 8$). #Significant differences ($p < 0.05$) between control mice (receiving saline) and rolipram-treated mice (IPN/6 wk/Prov/Rolipram). *Significant differences ($p < 0.05$) between control mice (receiving rat IgG) and anti-VLA-4 (IPN/6 wk/Prov/anti-VLA-4) and anti-IL-5 (IPN/6 wk/Prov/anti-IL-5)-treated mice.

ity as a contributing mechanism to the attenuation of AHR, we studied the effect of rolipram on MCh-induced responses in naive mice. Mice were administered rolipram (0.3 mg/kg) and lung function was evaluated. As shown in Figure 5, there were no significant differences between rolipram-treated mice and saline-treated mice at each concentration of MCh, indicating that the effects of rolipram were not attributable to a direct bronchodilating effect.

Treatment with Rolipram Inhibits Eosinophil, Lymphocyte, and Neutrophil Accumulation in BALF After OVA Provocation

To assess the effects of rolipram on the development of allergic inflammation after secondary challenge to OVA, inflammatory cell accumulation in BALF was measured. Administration of rolipram 0.01, 0.1, and 0.3 mg/kg significantly prevented the increases in eosinophil and lymphocyte numbers in BALF in a dose-dependent manner. Only the highest dose of rolipram (0.3 mg/kg) significantly inhibited the increase in neutrophil numbers in BALF (Figure 6).

Isolated lung lymphocytes were analyzed by FACS. Approximately 30 and 25% of the cells in mice receiving OVA challenge alone were T- and B-cells, respectively. Numbers of CD4⁺ T-cells in mice receiving 1% OVA provocation (secondary challenge) after initial OVA sensitization and challenge was significantly increased compared with nonsecondary challenged mice (Figure 7). Treatment with rolipram 0.3 mg/kg significantly suppressed this increase. In contrast, the frequency of cells expressing the surface markers CD8 and B220 did not change significantly between groups.

Treatment with Anti-VLA-4 and Anti-IL-5 Attenuates Changes in Airway Resistance But Does Not Affect Cdyn after Secondary OVA Exposure

We compared the effects of rolipram (0.3 mg/kg) with the effects of anti-VLA-4 and anti-IL-5 on airway responsiveness. Administration of anti-VLA-4 and anti-IL-5 significantly prevented the increases in RL when compared with control mice receiving rat IgG (Figure 8A). However, treatment with these antibodies had no significant effect on changes in Cdyn (Figure

8B); in contrast, treatment with rolipram 0.3 mg/kg prevented the changes in both RL and Cdyn after secondary challenge (Figures 8A and 8B).

Treatment with Anti-VLA-4 and Anti-IL-5 Decreases Eosinophil Numbers But Does Not Affect Lymphocyte and Neutrophil Accumulation in BALF after OVA Provocation

The number of inflammatory cells in BALF was determined 48 hrs after rechallenge with OVA. Administration of anti-VLA-4 and anti-IL-5 significantly inhibited accumulation of eosinophils in BALF compared with the rat IgG-treated group. Treatment with rolipram 0.3 mg/kg not only inhibited the number of eosinophils but also the number of lymphocytes and neutrophils in BALF (Figure 9).

Treatment with Anti-VLA-4 and Anti-IL-5 Decreases IL-5 Levels But Does Not Affect IL-4 Levels in BALF after OVA Provocation

After secondary OVA challenge, BALF IL-5 levels were similar to those seen immediately after primary challenge. Treatment with rolipram 0.3 mg/kg significantly inhibited IL-5 levels in the BALF after OVA reexposure. Administration of anti-VLA-4 and anti-IL-5 also significantly suppressed IL-5 levels.

IL-4 levels in BALF after secondary OVA challenge were significantly lower than after primary challenge. Administration of anti-VLA-4 and anti-IL-5 had no significant effect on IL-4 levels, whereas treatment with rolipram 0.3 mg/kg significantly lowered IL-4 levels. IFN- γ levels in BALF were unaffected by any of the treatments (Figure 10).

Treatment with Rolipram, Anti-VLA-4 and Anti-IL-5 Does Not Affect Serum Anti-OVA IgE Antibody Levels

Administration of rolipram (0.3 mg/kg), anti-VLA-4, and anti-IL-5 had no significant effects on serum anti-OVA IgE, anti-OVA IgG₁, and total IgE levels compared with 1% OVA provocation control group (Table 1).

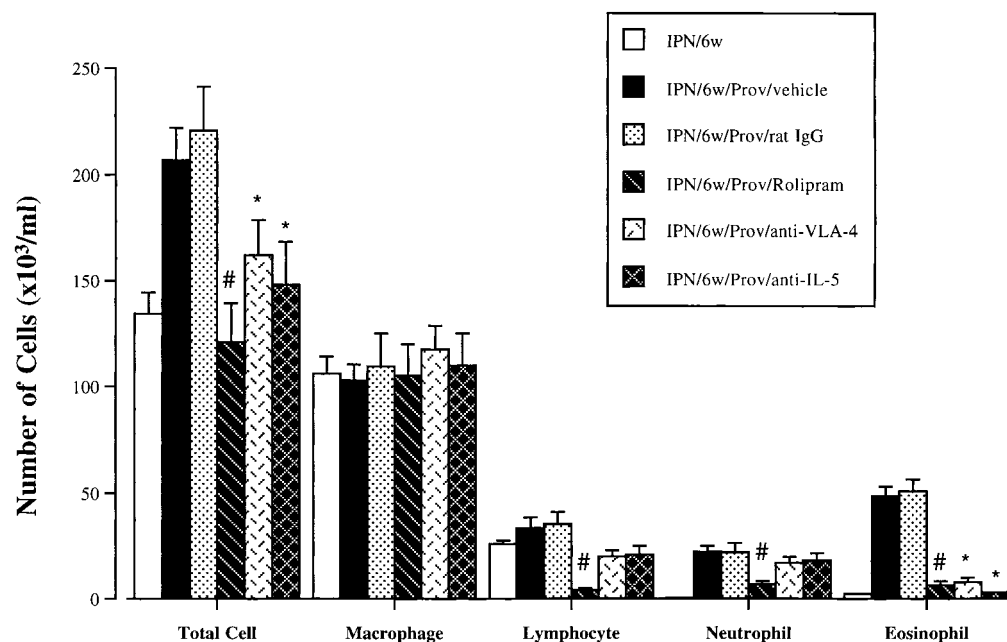


Figure 9. Anti-VLA-4 and anti-IL-5 significantly suppresses eosinophil accumulation but does not affect the number of lymphocytes and neutrophils in BALF after OVA provocation. Data represent the mean \pm SEM ($n = 8$). # Significant differences ($p < 0.05$) between saline-treated mice (IPN/6 wk/Prov/vehicle) and rolipram-treated mice (IPN/6 wk/Prov/Rolipram). *Significant differences ($p < 0.05$) between rat IgG-treated mice (IPN/6 wk/Prov/rat IgG) and anti-VLA-4 (IPN/6 wk/Prov/anti-VLA-4) and anti-IL-5 (IPN/6 wk/Prov/anti-IL-5) treated mice.

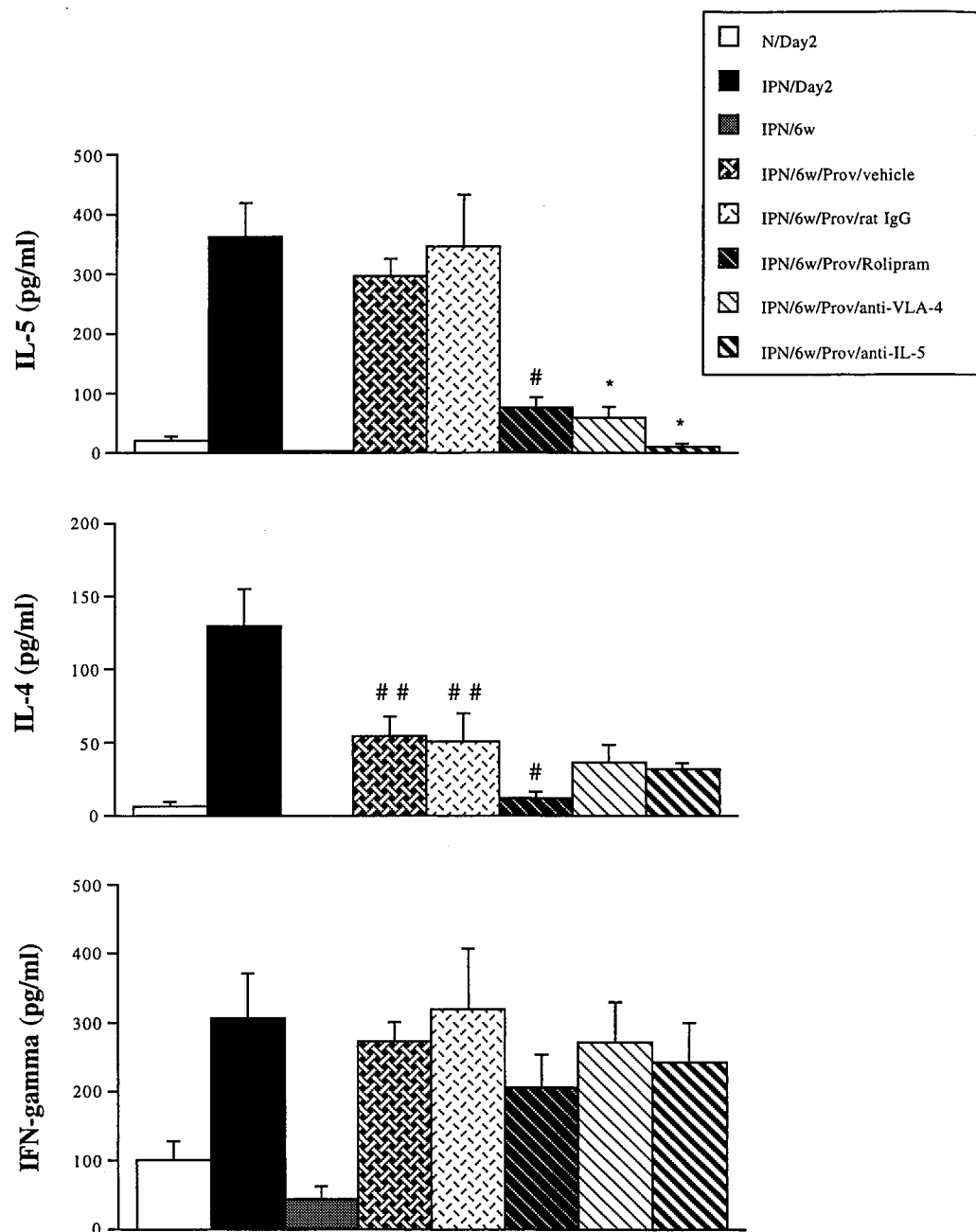


Figure 10. Effect of rolipram, anti-VLA-4 and anti-IL-5 on cytokine production in BALF of sensitized/challenged mice receiving a secondary challenge with OVA. IL-5, IL-4, and IFN- γ cytokine levels in BALF were assessed 48 h after allergen provocation as described in METHODS. The results for each group are mean \pm SEM (n = 8). #Significant differences (p < 0.05) between saline-treated mice (IPN/6 wk/Prov/vehicle) and rolipram-treated mice (IPN/6 wk/Prov/Rolipram). *Significant differences (p < 0.05) between rat IgG-treated mice (IPN/6 wk/Prov/rat IgG) and anti-VLA-4 (IPN/6 wk/Prov/anti-VLA-4) and anti-IL-5 (IPN/6 wk/Prov/anti-IL-5)-treated mice. ##Significant differences (p < 0.05) between sensitized and challenged mice (IPN/Day 2) and OVA-provoked control mice (IPN/6 wk/Prov/vehicle, IPN/6 wk/Prov/rat IgG).

Localization of Inflammatory Cells in Lung Tissue

The effects of the various treatments on tissue inflammatory cell infiltration were investigated using hematoxylin-eosin, PAS, and anti-MBP staining of lung sections. Secondary challenge to OVA via the airways increased the number of eosinophils, lymphocytes, and neutrophils in the peribronchial and perivascular tissues. Treatment with rolipram 0.3 mg/kg virtually abolished, and anti-VLA-4 or anti-IL-5 markedly reduced, the cellular infiltrates (Figure 11). Lung sections were stained with PAS in order to identify mucus containing cells in the airway epithelium. A large number of cells staining positive for mucus were found after secondary challenge. Surprisingly, only treatment with rolipram (0.3 mg/kg) inhibited PAS staining, whereas administration of anti-VLA-4 and anti-IL-5 had no significant effect on goblet cell mucus production (Figures 11 and 12). Further, staining with anti-MBP revealed the increase in eosinophils in the peribronchial and perivascular tissue af-

ter OVA reexposure as well and all three treatments suppressed eosinophil infiltration in the lung (Figure 13).

DISCUSSION

Cyclic AMP is an important second messenger involved in the regulation of immune and inflammatory cell function. Intracellular cAMP levels reflect the balance between the generation of cAMP from ATP by adenylate cyclase and its degradation by PDE. In particular, PDE4 has been identified in many inflammatory cells such as eosinophils, T-cells, and neutrophils (15). Consequently, PDE4 inhibitors may have a potential therapeutic intervention in allergic disorders such as asthma since eosinophils and T-cells have been closely correlated with disease activity in human and animal models of asthma. However, the exact mechanisms by which PDE4 inhibitors may attenuate allergen-induced airway hyperresponsiveness have not been fully defined.

TABLE 1
OVA-SPECIFIC ANTIBODY AND TOTAL IgE LEVELS IN THE SERUM*

	OVA-specific Antibody Levels (EU/ml)		Total IgE Levels (ng/ml)
	IgE	IgG ₁	
N/Day 2	35.26 ± 6.57	ND	196.84 ± 19.68
IPN/Day 2	225.02 ± 8.1 [§]	16.12 ± 2.24 [§]	506.53 ± 98.12 [§]
IPN/6 wk	62.82 ± 7.6	6.54 ± 0.67	239.99 ± 63.28
IPN/6 wk/Prov vehicle	129.8 ± 8.3 ^{†‡}	12.58 ± 2.34 [†]	473.62 ± 50.66 [†]
IPN/6 wk/Prov rat IgG	101.54 ± 15.37 ^{†‡}	14.64 ± 4.56 [†]	458.64 ± 85.79 [†]
IPN/6 wk/Prov Rolipram	108.94 ± 11.58	11.41 ± 0.8	390.53 ± 25.17
IPN/6 wk/Prov anti-VLA-4	116.74 ± 10.93	13.3 ± 1.47	429.78 ± 43.68
IPN/6 wk/Prov anti-IL-5	125.94 ± 9.91	11.64 ± 1.72	394.49 ± 51.08

* Serum levels of OVA-specific antibodies and total IgE levels were determined by ELISA as described in METHODS (ND = not detectable). Data represent the mean ± SEM (n = 8).

[†] Significant differences (p < 0.05) between OVA provocation control mice (IPN/6 wk/Prov/vehicle or IPN/6 wk/Prov/rat IgG) and mice without provocation (IPN/6 wk).

[‡] Significant differences (p < 0.05) between OVA provocation control mice (IPN/6 wk/Prov/vehicle or IPN/6 wk/Prov/rat IgG) and sensitized and challenged mice (IPN/Day 2).

[§] Significant differences (p < 0.05) between OVA-challenged alone (N/Day 2) and OVA-sensitized and challenged mice (IPN/Day 2).

In this study, we used a model of allergen-induced AHR after reexposure in previously sensitized mice to more closely resemble human disease (1) and not simply examine effects on primary responders. The effects of a specific PDE4 inhibitor,

rolipram, on airway function was monitored by changes in lung function to inhaled MCh and the results were compared with administration of anti-IL-5 or anti-VLA-4. Airway challenge of sensitized BALB/c mice triggered significant in-

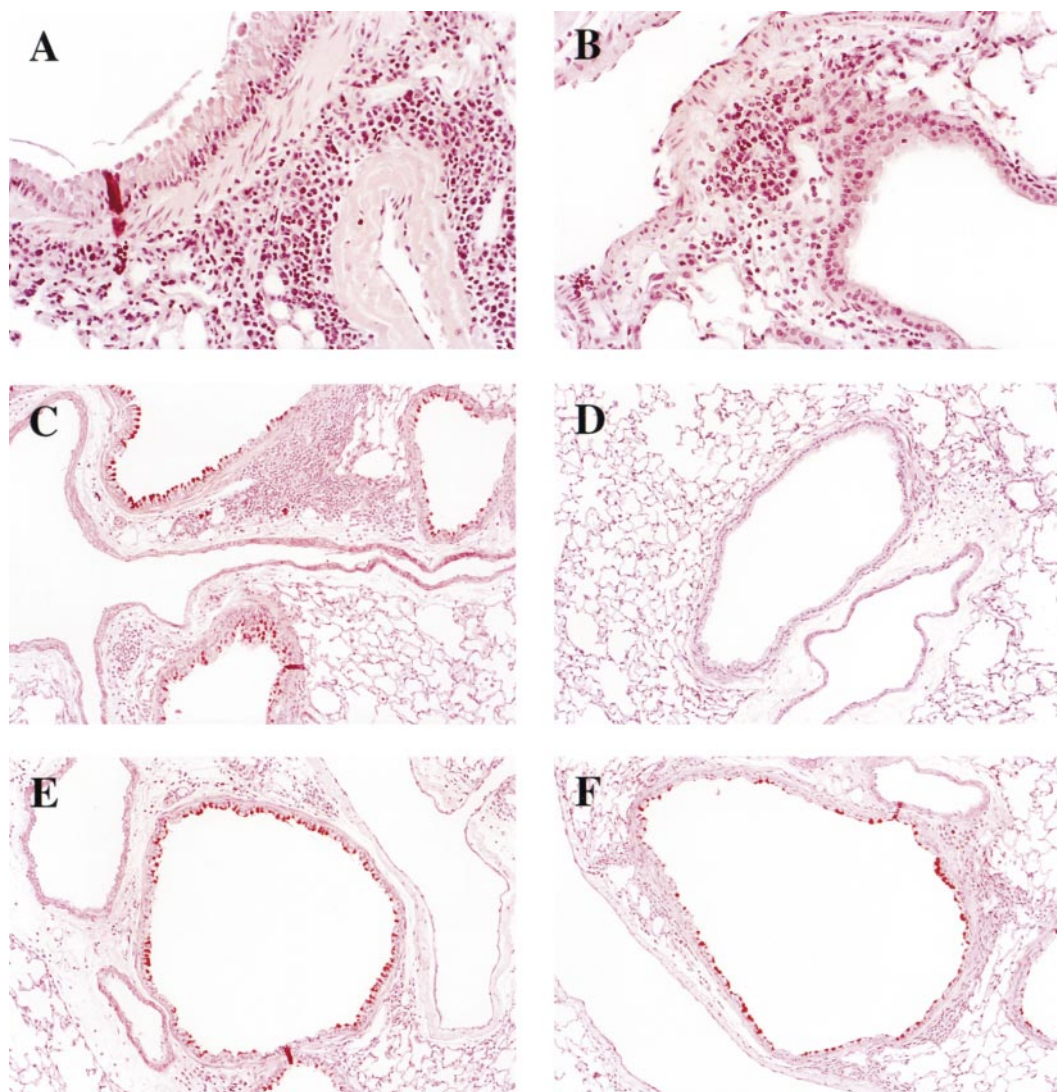


Figure 11. Inflammatory cell response. Evidence of inflammatory cell infiltration was investigated by histologic examination of hematoxylin-eosin-stained tissue as described in METHODS (final magnification: ×200). (A) Two days after the last challenge after intraperitoneal sensitization (IPN/Day 2); (B) 1% OVA provocation after OVA sensitization and challenge (IPN/6 wk/Prov/rat IgG). Histologic examination of lung sections stained with PAS in order to identify mucus-containing cells in the airway epithelium as described in METHODS (final magnification: ×200); (C) 1% OVA provocation after OVA sensitization and challenge (IPN/6 wk/Prov/rat IgG); (D) treatment with rolipram (IPN/6 wk/Prov/Rolipram); (E) treatment with anti-VLA-4 (IPN/6 wk/Prov/anti-VLA-4); (F) treatment with anti-IL-5 (IPN/6 wk/Prov/anti-IL-5).

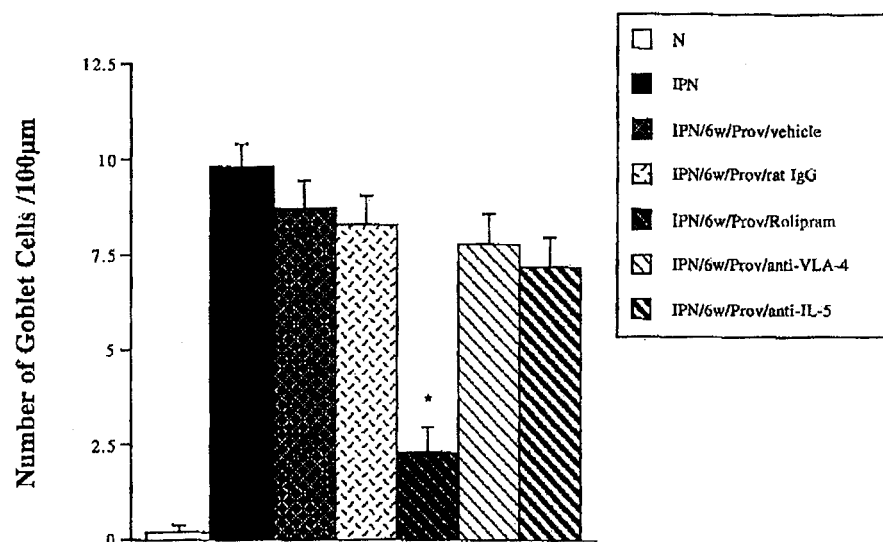


Figure 12. Treatment with rolipram prevents goblet cell hyperplasia. Numbers of mucus-containing cells in the airway epithelium (number of goblet cells/100 μ m epithelium) were quantitated by histologic examination of PAS-stained tissue as described in METHODS. The results for each group are mean \pm SEM ($n = 8$). *Significant differences ($p < 0.05$) between sensitized and challenged control mice (IPN/6 wk/Prov/vehicle) and sensitized and challenged, rolipram-treated mice (IPN/6 wk/Prov/rolipram).

creases in RL and reductions in Cdyn to inhaled MCh in a dose-dependent manner. The changes in RL and Cdyn and the number of eosinophils in BALF reached maximal levels 48 h (2 d) after the last challenge, and persisted for at least 14 d after OVA challenge. By 6 wk, the changes in RL and Cdyn and the numbers of eosinophils in BALF and in the tissues were restored to baseline levels. Interestingly, only the number of lymphocytes in BALF continued to increase at 6 wk. Secondary challenge to allergen at this time point triggered changes in RL and Cdyn, and increased IL-4 and IL-5 levels in BALF. The number of lymphocytes (CD4+ T-cells) and neutrophils in BALF also significantly increased. Furthermore, the number of MBP+ cells in the lung tissue after OVA provocation was the same as 2 d after the last of the primary challenges, despite the finding that numbers of eosinophils in BALF were lower. In addition, higher anti-OVA IgE levels were detected after OVA provocation. These data establish that secondary challenge to allergen in previously sensitized/challenged (but not in nonsensitized) mice elicits eosinophil, neutrophil, and lymphocyte accumulation in the lung as well as altered airway function. These differences in the composition of the inflammatory cell infiltrate after primary versus secondary challenge may underlie, to some extent, the differences in response to different therapeutic interventions.

Under these conditions of secondary challenge in allergic mice, intraperitoneal administration of rolipram significantly prevented increases in lung resistance and decreases in Cdyn and inflammatory cell accumulation in a dose-dependent manner. The accumulation of eosinophils as well as the increase in CD4+ lymphocytes and neutrophils was inhibited by the drug, and levels of IL-4 and IL-5 in the BALF were significantly lower. The doses of anti-VLA-4 and anti-IL-5 were previously shown to be optimal after primary challenges (3, 6). Further, doubling the administered dose of antibody was not more effective in the secondary challenge model (data not shown). In contrast to rolipram, anti-VLA-4, although effective in preventing increases in RL and eosinophil infiltration, had little effect on Cdyn, lymphocyte, or neutrophil numbers or levels of IL-4. Similarly, anti-IL-5 administered 2 h before OVA provocation, was only effective in attenuating changes in airway resistance and inhibiting eosinophil inflammation without affecting Cdyn, lymphocyte, or neutrophil numbers or IL-4 levels.

These differences are quite striking in that anti-VLA-4 or anti-IL-5 have been shown to be very effective in preventing

changes in both RL and Cdyn when administered prior to primary OVA challenge (3). However, delaying administration of anti-VLA-4 to a time point after primary OVA challenge completely eliminated the ability of the antibody to prevent changes in Cdyn, although it was still effective in attenuating RL and prevented eosinophil inflammation (3). In a similar fashion, anti-IL-5 has been shown to prevent changes in RL and inhibit eosinophil inflammation, even in previously sensitized and challenged mice reexposed to OVA, but changes in Cdyn were not susceptible (unpublished data).

Cumulatively, these data dissociating RL and Cdyn as well as the responses to primary versus secondary challenge suggest that development of changes in lung function assessed by measurements of airway resistance and dynamic compliance may be under different regulatory control. Changes in RL are thought to reflect alterations in the function of larger or central airways, whereas Cdyn may represent small or peripheral airway function (22–24). On the basis of previous data with anti-VLA-4 (3) and the present data, it appears that eosinophils may be more important to the development of changes in RL as all three approaches, rolipram, anti-IL-5, and anti-VLA-4, prevented eosinophils from accumulating in the lung and this was associated with normalization of lung resistance. In contrast, only rolipram prevented changes in Cdyn. Whether this can be linked to the concomitant reduction in lymphocyte and neutrophil numbers or goblet cell hyperplasia remains to be determined.

The doses of rolipram that were used in the present study were lower than those used in other lung inflammation models in mice, but they were similar to those employed in guinea pigs (9, 25, 26). It has been shown that PDE4 inhibitors have a bronchodilatory effect on human airway smooth muscle (21). To ensure that this possibility was not a contributing mechanism to the attenuation of AHR, we evaluated the effect of rolipram on MCh-induced increase in airway resistance in naive mice. Rolipram had no significant effect on airway function under these conditions.

The precise mechanism whereby PDE4 inhibitors attenuate allergen-induced airway hyperresponsiveness and inflammation is not known. PDE4 inhibitors have proven effective in guinea pig (9) and monkey models (11) of AHR and eosinophilia. PDE4 inhibitors can reduce the activation of eosinophils *in vivo* as assessed by decreased eosinophil peroxidase release into BALF (27) and the downregulation of antigen-

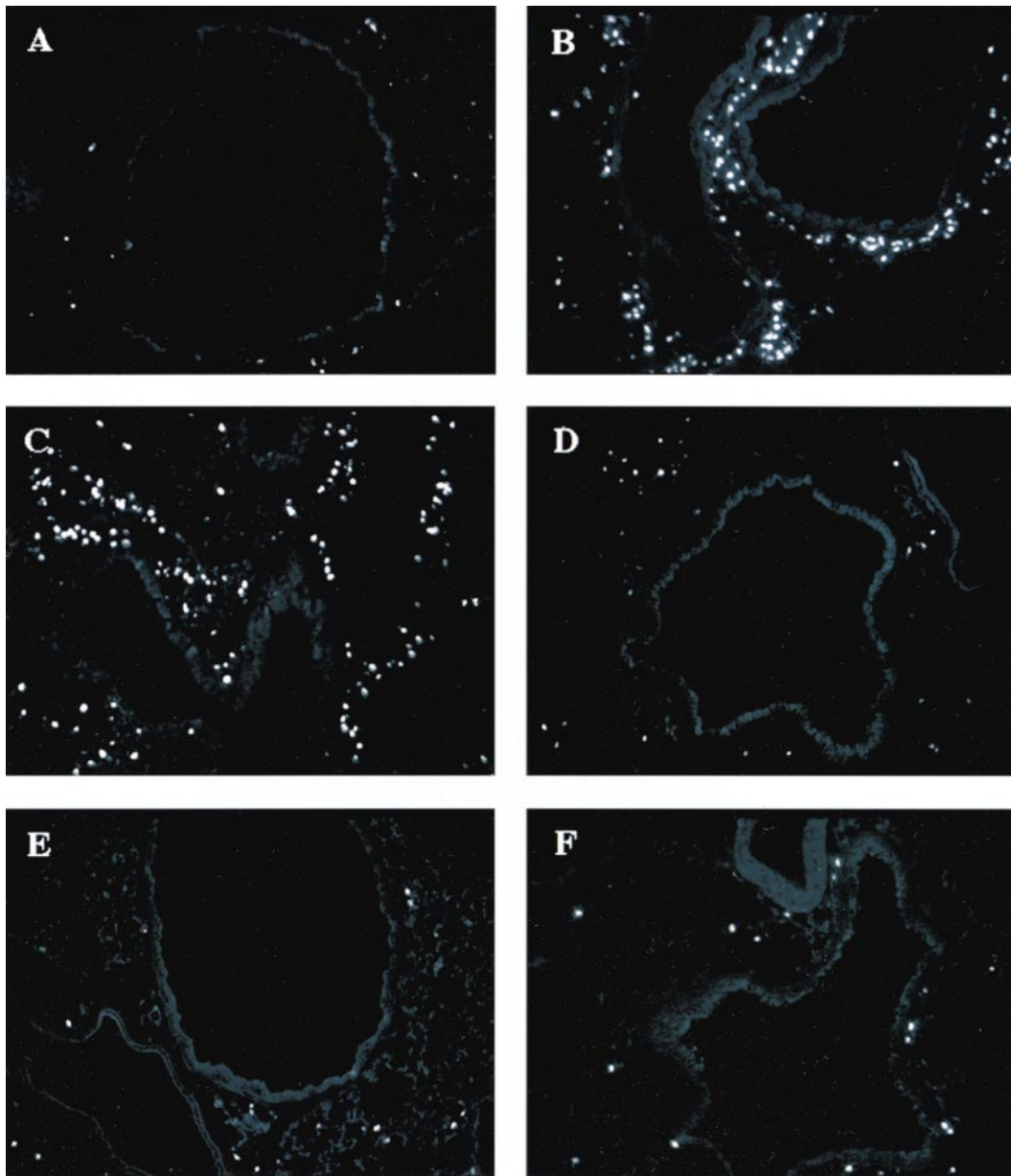


Figure 13. Immunohistochemistry of peribronchial and perivascular tissue. A rabbit antimouse MBP antibody and a fluorescein-labeled goat anti-rabbit IgG was used as described in METHODS (final magnification: $\times 200$). (A) Six wk after the last challenge (without provocation) (IPN/6 wk); (B) 2 d after the last challenge after OVA sensitization (IPN/Day 2); (C) 1% OVA provocation after sensitization and challenge (IPN/6 wk/Prov/rat IgG); (D) treatment with rolipram (IPN/6 wk/Prov/Rolipram); (E) treatment with anti-VLA-4 (IPN/6 wk/Prov/anti-VLA-4), (F) treatment with anti-IL-5 (IPN/6 wk/Prov/anti-IL-5).

induced IL-5 gene expression and protein (28). Moreover, rolipram suppressed PAF- and C5a-stimulated LTC₄ synthesis in human eosinophils from atopic subjects (13). The pulmonary vasculature and endothelium are other potential sites of action since endothelial cells express PDE4, and increased levels of cAMP in these cells have been reported to reduce the expression of VCAM-1, which plays an important role in the recruitment of eosinophils into the airways (17).

One possible target of rolipram is the T-cells, as this study showed that only rolipram significantly prevented the increase in CD4⁺ T cell accumulation and IL-4 levels in the BALF, 48 h after OVA provocation. Furthermore, using a PAS stain to monitor mucus production, we saw increases in goblet cell staining after OVA sensitization and challenge and after OVA provocation. Only treatment with rolipram had an in-

hibitory effect on mucus production. We previously showed that IL-4 may be essential for mucus production in sensitized and challenged mice (29), and induction of goblet cell hyperplasia might be an important factor in the development of altered dynamic compliance (3, 24, 25, 30). The suppressive effect of rolipram on IL-4 production in the airways might modulate the changes in the epithelium of peripheral airways and goblet cell hyperplasia. Neutrophils may also represent an important target for modulation by PDE4 inhibitors, because PDE4 is the predominant cAMP-metabolizing enzyme in human neutrophils (16, 31). Similar to the effects on eosinophil influx into the airways, PDE4 inhibitors markedly reduced antigen-stimulated neutrophil infiltration in several animal models (9, 11, 12). In peripheral blood neutrophils, PDE4 inhibitors reduced superoxide anion production in response to a number

of stimuli, including FMLP, C5a, and GM-CSF (16, 32). Other neutrophil functions regulated by PDE4 inhibitors include leukotriene production and adhesion to endothelial cells (31, 33). Miotla and colleagues (25) have demonstrated that rolipram suppressed neutrophil sequestration in pulmonary capillaries and lung myeloperoxidase activity in acute lung injury models in mice.

In summary, these studies identify the potential of PDE4 inhibitors in allergic inflammation and airway hyperresponsiveness. Although the mechanism of action can only be speculated upon, in comparison with known reagents such as anti-VLA-4 or anti-IL-5, rolipram not only was comparable but appeared to have added benefit in allergic mice rechallenged with allergen. At a minimum, the results indicate that certain aspects of lung function, in particular central versus peripheral airway function and goblet cell hyperplasia, may be differentially regulated and must be considered when novel therapies are introduced. In this regard, a PDE4 inhibitor may have advantages over antagonists of VLA-4 or IL-5.

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